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TITLE: The Preclinical Evaluation of Fever-Range, Whole Body Hyperthermia as an Adjuvant to Chemotherapy and Cytokine Immunotherapy for the Treatment of Breast Cancer

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<b>13. ABSTRACT (Maximum 200 Words)</b> The work outlined in the original grant proposal was designed to develop new breast cancer treatments that combined a current breast cancer chemotherapy (doxorubicin and Doxil) and a cytokine-based immunotherapy (IL-12 and IL-2) with fever-like whole body hyperthermia (WBH). We have found that the combination of WBH with Doxil does indeed result in a better anti-tumor effect in our SCID mouse human breast tumor xenograft model when compared to Doxil alone. Experiments examining free Doxorubicin are underway. Additional work has shown that free IL-12 when used in combination with WBH only marginally enhances the anti-tumor response when compared to the IL-12 alone in our model. We feel that this is due to the lack of T and B cells in the SCID mice that may have inhibited our cytokine immunotherapy protocol. Therefore additional experiments have been executed using a different model (BALB/c mice bearing Colon 26 tumors) while a more appropriate breast cancer model can be developed. Data using this model have been extremely promising when using both free IL-12 and polylactic acid microsphere encapsulated IL-12. Overall, the results generated the past year have been promising.							
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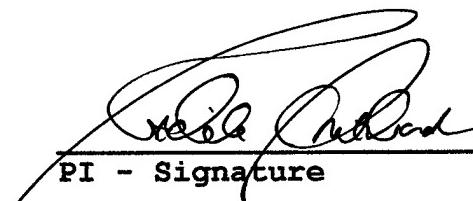
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Dr. Richard  
PI - Signature

29 Jun 2000  
Date

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## **INTRODUCTION**

The work outlined in the original grant proposal was designed to develop better breast cancer treatments that combined liposome encapsulated Doxorubicin (Doxil) and microsphere encapsulated cytokines (IL-2 and IL-12) with fever-like whole body hyperthermia (WBH) and to compare these treatments with those of free drug + WBH. WBH has been shown by our lab to have an anti-tumor effect when used alone. Unfortunately, WBH does not result in the resolution of disease in any of our mouse models. We hypothesize that our dual modality approach will enhance the efficacy of either WBH and chemotherapy or cytokine immunotherapy alone. In the past year, we have found that the combination of WBH with Doxil does indeed result in a better anti-tumor effect in our SCID mouse human breast tumor xenograft model when compared to Doxil alone. Additional work has shown that free IL-12 used in combination with WBH only marginally enhances the anti-tumor response when compared to the IL-12 alone in our model. We feel that this is due to the lack of T and B cells in the SCID mice and therefore may have inhibited the efficacy of this cytokine immunotherapy protocol. Additional experiments have been performed using an immunocompetent mouse model and have yielded positive results. Overall, the data generated in the past year have been exciting as well as promising for continued success of the goals outlined in the Statement of Work.

## BODY

**Task I:** To determine if a synergy exists in the anti-tumor effects of WBH in combination with chemotherapeutic agents using specialized microparticle delivery systems.

The SCID mouse/ human tumor xenograft model has been extremely useful in determining the anti-tumor efficacy of Doxil used in combination with WBH. Tumors for these experiments were originally derived from patients without prior chemotherapy. This strategy allowed us to alleviate a potential pitfall prior to its inception. It is widely believed that tumors from patients who had received chemotherapy could express P-glycoprotein (pgp). Pgp is the product of the multi-drug resistance gene and, as its name suggests, its presence has been linked to tumor resistance of chemotherapeutic drugs.

The first experiment was designed to answer the following question: would the combination of WBH and Doxil have a greater anti-tumor effect than either modality alone in SCID mice bearing human breast tumor #8990? Small pieces (2 x 2 mm) of human breast tumor, derived from a passage of that tumor growing a SCID mouse, were implanted subcutaneously (s.c.) in the lower left abdominal quadrant. After two weeks, when the tumors reached a size of 5 x 6 mm, the treatment regime began. Unfortunately, this tumor did not grow in each mouse into which it was implanted, limiting the number of mice of the experiment. Therefore, only 3 groups of mice were used: a control group, a Doxil alone group and a Doxil and WBH group and each group contained 5 mice. We omitted the WBH group for many experiments have been performed with this control and the anti-tumor response is always slight. Obviously we anticipated repeating this experiment with this control immediately subsequent to this unfortunate occurrence.

Doxil, provided by Alza Pharmaceuticals, was given weekly by tail vein (i.v.) injection at 2.0 mg/kg in a 100 $\mu$ Ls for 4 weeks. Control mice were given the same volume of sterile saline i.v. WBH treatment was given twice, once on day 2 and then again on day 10. Both WBH treatments were scheduled such that they occurred 2 days after the Doxil treatment. The tumors in these animals were measured periodically during the course of the treatment schedule. In addition to the tumor measurements, the mice were observed daily to assess general health. Notably, there was no evidence of drug related toxicity (as evidenced by weight loss, posture and coat cleanliness) in the mice. Animals were sacrificed when their tumors reached 2.0 cm in any diameter.

The data collected from this experiment were plotted in two ways: First as a kinetic graph examining the average relative growth of tumors per group, and second, as a graph that examines the time it took for the tumor in each mouse to reach 2.0 cm in diameter. The former method allows us to directly examine the effect of our treatment on tumor size

over time while the latter method allows us to examine the potential for our treatment to lengthen survival of our mice (Kaplan-Meier plot). We do not want our animals to suffer as a result of these experiments, so we chose a tumor size of 2.0 cm to act as a suitable endpoint instead of waiting until the mouse succumbed to its disease.

The kinetic data (Figure 1 A) were not all that impressive as the tumor growth curves for the Doxil alone and Doxil + WBH are similar out to day 20. It was at this point that the tumors in mice of the control began approaching the 2.0 cm diameter limit. However, the Kaplan-Meier plot (Figure 1 B) illustrates that the combination treatment resulted in a survival advantage for these mice well past day 20. Sixty percent of tumors reached 2.0 cm in the control group by day 28, by day 40 in the Doxil alone group and by day 56 in the combination group.

At the same time these results were analyzed, other work by the trainee was confirming a distinct enlargement in tumor blood vessels up to 2 weeks post WBH (Figure 2). We hypothesize that tumor infiltrating blood vessels are unable to contract post WBH due to their lack of surrounding smooth muscle cells and pericytes. This important observation was taken into consideration in the design of the second experiment.

The second experiment was designed to ask two different questions: Is the combined effect of Doxil + WBH on tumor growth and survival tumor specific? And, is it possible to optimize the treatment schedule for a greater anti-tumor effect? In this case, we used human breast tumor #10366 that was currently growing in a SCID mouse.

Unfortunately, we once again had poor tumor growth despite the fact that extra mice were implanted with this tumor to control for our disappointing tumor engraftment rate in the previous experiment. Five animals were in each of the following groups: control, Doxil alone and Doxil + WBH.

Interestingly, as we have noted in many other human tumor xenografts, the growth rate of this tumor differed considerably from tumor #8990; #10366 grew more slowly. This exemplifies one of the advantages in using the SCID mouse system for it closely reflects the heterogeneous growth characteristics of human tumors.

In this experiment, we performed WBH treatment 6 times in conjunction with 3 Doxil treatments (2 mg/kg); two WBHs and 1 Doxil treatment weekly for three weeks. Our rationale was as follows: use the first WBH to increase the blood vessel size to facilitate entry of the Doxorubicin loaded liposomes into the tumor and the second WBH 2 days after the first to increase the release kinetics of the Doxorubicin from the liposome lodged in the tumor.

Figure 3 illustrates the tumor growth kinetics in this experiment. The

Doxil treatment was remarkably enhanced with the application of the WBH treatment. These data were extremely exciting and have been instrumental in designing a Phase I/II trial in breast cancer patients at Roswell Park Cancer Institute examining the combination of Doxil and WBH.

Work on Task I allowed the trainee to become quite skilled in various injection techniques, animal handing and surgical techniques.

Ongoing and future work will concentrate on repeating these experiments with special attention to mechanism behind the observed anti-tumor response. In addition, we wish to compare Doxil to free Doxorubicin when used in combination with WBH. Delay in this regard was due to difficulty in obtaining the free drug. In addition, the tumor specimens will be analyzed for pgp expression at the completion of all these studies.

**Task II:** To determine if a synergy exists in the anti-tumor effects of WBH in combination with cytokine immunotherapy agents administered using specialized microparticle delivery systems.

In the year between submitting and announcement of the funding of this pre-doctoral training grant, work on Task II had commenced. Free IL-12 became available from Genetics Institute earlier than it was expected.

During that year many SCID mouse/ human tumor xenograft experiments had been performed combining WBH with many different cytokines. In each of these experiments a sub-optimal cytokine dosage was used so that an enhancement of its anti-tumor efficacy could be analyzed by combination with WBH. In each and every circumstance, the cytokine was so successful on its own that its combination with WBH did not result in a greater anti-tumor effect when compared to the cytokine alone. When the lowest dosages of cytokine were tested, a lack of any anti-tumor effect was noted and this lack of effect could not be enhanced by WBH.

This work lead to one experiment exploring the combination of IL-12 with WBH in SCID bearing human breast tumor #8990. We designed the experiment to ask the following question: which dosage of cytokine can be enhanced by WBH? The animals were placed in one of 10 groups containing 2 mice each. Fifty, 100, 200 and 400 ng of IL-12 per mouse per day were used in combination with WBH.

As shown in Figure 4, the anti-tumor effect of IL-12 at a dosage of 100 ng was slightly enhanced by WBH. This dose is 1/3 that of the optimal dose of 300 ng used in other experiments in the lab. It is recognized that the observed enhancement is slight, and due to the small number of animals we were unable to determine statistical significance of this work. However, due to the many other experiments that had been performed in non-breast tumor models that resulted in the same, albeit statistically insignificant, trend, we felt somewhat encouraged that

this dosage showed the greatest potential for further studies.

Unfortunately, when the data were analyzed further by other members of the lab, it was determined that the anti-tumor effect of the combination treatment in this model was not effective enough to study further. The utility in using the SCID mouse/human breast tumor xenograft model in testing cancer immunotherapy seemed to pale in comparison to that in testing chemotherapeutic treatment regime. We hypothesize that the lack of a complete immune system in the SCID mouse model curtailed the effectiveness of this treatment protocol.

Until a more suitable breast tumor model could be developed, we examined the effect of WBH in combination with either IL-12 alone or IL-12 delivered in polylactic acid microspheres in a well known colon tumor model: Colon 26 (CT26) which is syngeneic in BALB/c mice. Our rationale was as follows: if success could be achieved in this immunocompetent mouse model, then the chances of success in a breast tumor model also in an immunocompetent mouse would be highly probable.

Many experiments have been performed exploring this idea. The first asked the question: Is there an enhancement of the anti-tumor response when combining IL-12 with WBH? To answer this question,  $1 \times 10^5$  CT26 cells were implanted s.c. in the lower left abdominal quadrant of female BALB/c mice. Ten days later (day 0), the tumors were measured and the animals were placed into 4 groups with 5 mice each. On day 1, WBH was performed. After the WBH, the mice resumed normal body temperature in 15 minutes and were given their first dose of IL-12, 100 ng, i.p. IL-12 treatment continued for 14 days. Tumors were measured periodically over the next 49 days. Figure 5 A shows the tumor growth over time. Unfortunately, no significant difference in tumor growth was observed between the IL-12 alone and the IL-12 + WBH groups up to Day 13. However, as seen in Figure 5 B, when tumors were allowed to grow to a size of  $1500\text{mm}^3$  before the mice were sacrificed, a survival advantage was noted in mice given the combination treatment. In fact, one animal in this group was cured of its disease. Although common in mouse models of cancer, the resolution of disease in this particular animal was extremely exciting for it was the first time the trainee had ever achieved such a success.

The subsequent experiment wished to answer the following questions: What is the best placement of the WBH with respect to the initiation of the IL-12 treatment? Could it be possible to enhance the anti-tumor response by simply altering the spatial distribution of the two treatments? Once again, BALB/c mice bearing s.c. CT26 were placed into 6 groups of 5 mice each. The groups were as follows: control, WBH, IL-12, WBH + IL-12 pre (immediately before WBH), WBH + IL-12 post (15 minutes after WBH), WBH + IL-12 (12 hours post WBH). One-hundred ng of IL-12 was administered per mouse, i.p. for 14 days beginning with the first dose as described above.

As illustrated in Figure 6 A, there is very little difference in the overall tumor growth between any of the IL-12 treated groups up to Day 12. Once again however, when the mice were followed out past the cessation of IL-12 treatment and sacrificed when their tumors reached a volume of 1000mm<sup>3</sup>, a survival advantage was noted in the group that received its first IL-12 dose (15 minutes) post WBH (Figure 6 B). In fact, this treatment scheme appeared to be the best regime for three reasons: 1. This was the only group that had 100% of animals with tumors <1000mm<sup>3</sup> to day 30, 2. This was the only group with 2 cures, and 3. On the last day of the experiment (Day 43) the remaining mouse with a tumor in this group had the smallest volume (just over 1000mm<sup>3</sup>) while the other tumors were all very close to 1500mm<sup>3</sup>.

Interestingly, if IL-12 is given immediately before WBH a decrease in survival was noted; all animals in this group were sacrificed by Day 30. This was an unexpected result, one that we are quite fortunate to have discovered prior to implementation of this sort of immunotherapy protocol in a clinical trial. In addition, the mice in this particular group exhibited the most drastic signs of drug-related toxicity (labored breathing, hunched appearance, ruffled fur and weight loss).

In the most recent experiment, we wished to ask the following question: do encapsulated cytokines yield a greater anti-tumor response when used in combination with WBH? On Day 1, female BALB/c mice bearing CT26 tumors were treated with WBH after which, a single intratumoral injection of IL-12 microspheres (or BSA loaded microspheres as a control) was given. Tumors were measured periodically over time. Figure 7 illustrates the amazing anti-tumor effect of IL-12 loaded microspheres + WBH. Four of 5 tumors in this group ceased to grow after the combined treatment and yielded 3 durable cures. Interestingly, there was a complete lack of drug related toxicity using the microsphere encapsulated IL-12 in any group when compared to the toxicity the mice exhibited when treated with free IL-12.

The training value of this body of work was invaluable. The experiments performed initially yielded disappointing results. However, this was important information and changes were made to test the hypothesis further in another, more appropriate mouse model. The experience and insight gained by the trainee is recognized as vital to future experimental design.

We hope that a better breast tumor model will be developed with which to study our immunotherapy protocol. Histological analysis of tumors will be completed as will an assessment of immune cell infiltrate into those tumors. In addition, experiments will be completed which examine the anti-tumor effect of another cytokine, IL-2 both in free drug and encapsulated formulations, when used in combination with WBH.

## **KEY RESEARCH ACCOMPLISHMENTS**

1. An enhancement of the anti-tumor effect of Doxil when used in combination with WBH was achieved in the SCID mouse/ human breast tumor xenograft model.
2. The treatment regime was optimized to a degree. However, it is recognized that it can be further improved for cures were not achieved in any of the test subjects.
3. A slight, yet statistically insignificant enhancement of the anti-tumor effect of cytokine immunotherapy was achieved when used in combination with WBH in the SCID mouse/human breast tumor xenograft model. However, a much greater anti-tumor effect of cytokine immunotherapy + WBH was noted when using a mouse model with a complete immune system.
4. Importantly, it was recognized that a single mouse model, although sufficient for some experiments, cannot be the best model with which to test all experimental treatments. Therefore, the ability to recognize and flexibility to deal with such setbacks are essential qualities for success in a graduate training program.
5. Finally, there appear to be distinct benefits to using microsphere encapsulated IL-12. They include the following: only one treatment was given compared to 14 treatments with the free cytokine and the treatment was delivered directly into the tumor compared to the free cytokine which was delivered systemically. These two points seem to have lead to a decrease in drug related cytotoxicity in the IL-12 microsphere treated mice compared to that of the mice that were treated with free drug. These points could have tremendous clinical impact for human cancer patients.

## **REPORTABLE OUTCOMES**

Only one Departmental seminar was given in the past year that included approximately half of the year's data.

No abstracts or manuscripts have been submitted.

No patents or licenses have been applied for or received

No degrees have yet been obtained that are supported by this award

No new cell lines have been developed. Tissue and serum repositories have been collected for each experiment for future analysis.

No informatics have been developed.

No funding has been applied for based upon the work supported by this award.

No employment or research opportunities have been applied for or received based upon experience or training supported by this award.

## CONCLUSIONS

The conclusions will be described in conjunction with each figure found in the Appendix.

**Figure 1 A.** The overall anti-tumor effect of Doxil + WBH was very slight when human breast tumor growth was plotted over time out to Day 20. We concluded that our treatment protocol would need to be optimized.

**Figure 1 B.** A survival advantage was observed in mice from the combination group compared to that of the Doxil alone group when tumor size was analyzed out to Day 69. It is interesting to note that this effect may have been overlooked provided that the mice were simply sacrificed all at once instead of waiting until their tumors reached 2.0 cm in diameter.

**Figure 2.** Tumor blood vessels expand due to WBH and are unable to contract again for at least 2 weeks. We conclude that this may be an important mechanism by which large encapsulated drugs can gain access to the tumor and thereby have a greater anti-tumor effect.

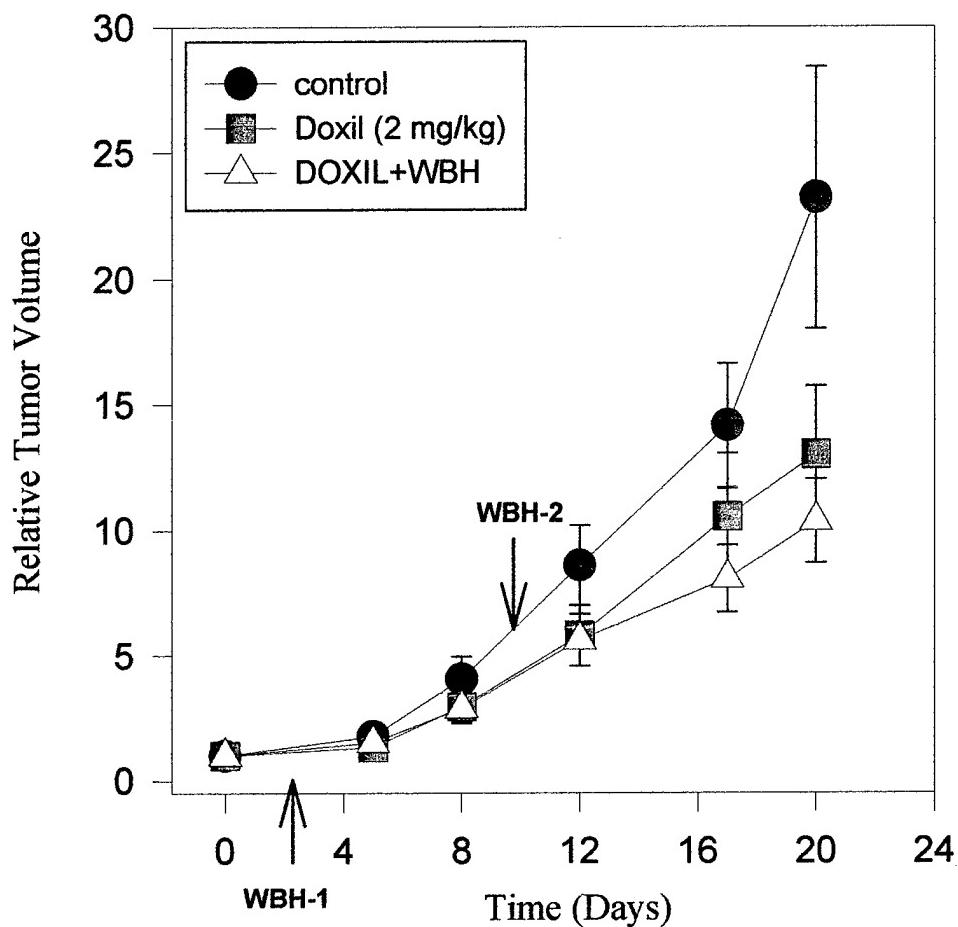
**Figure 3.** By learning from previous experiments, a new experiment was designed with the goal of optimizing the WBH-chemotherapy treatment protocol. The WBH-Doxil-WBH protocol resulted in a tremendously exciting anti-tumor response in the combination group when compared to that of the Doxil alone group. We conclude that timing of WBH with respect to treatment with Doxil is an extremely important parameter to consider.

**Figure 4.** This was a preliminary experiment with only 2 mice per group. It did, however, confirm a trend we had previously observed in many other SCID experiments. A slightly better effect of 100 ng of IL-12 used in combination with WBH compared to that with IL-12 alone was shown. Unfortunately, due to the lack of statistically significant results in any of these SCID mouse human tumor xenograft experiments we concluded that a model involving an immunocompetent mouse must be explored for future immunotherapy work.

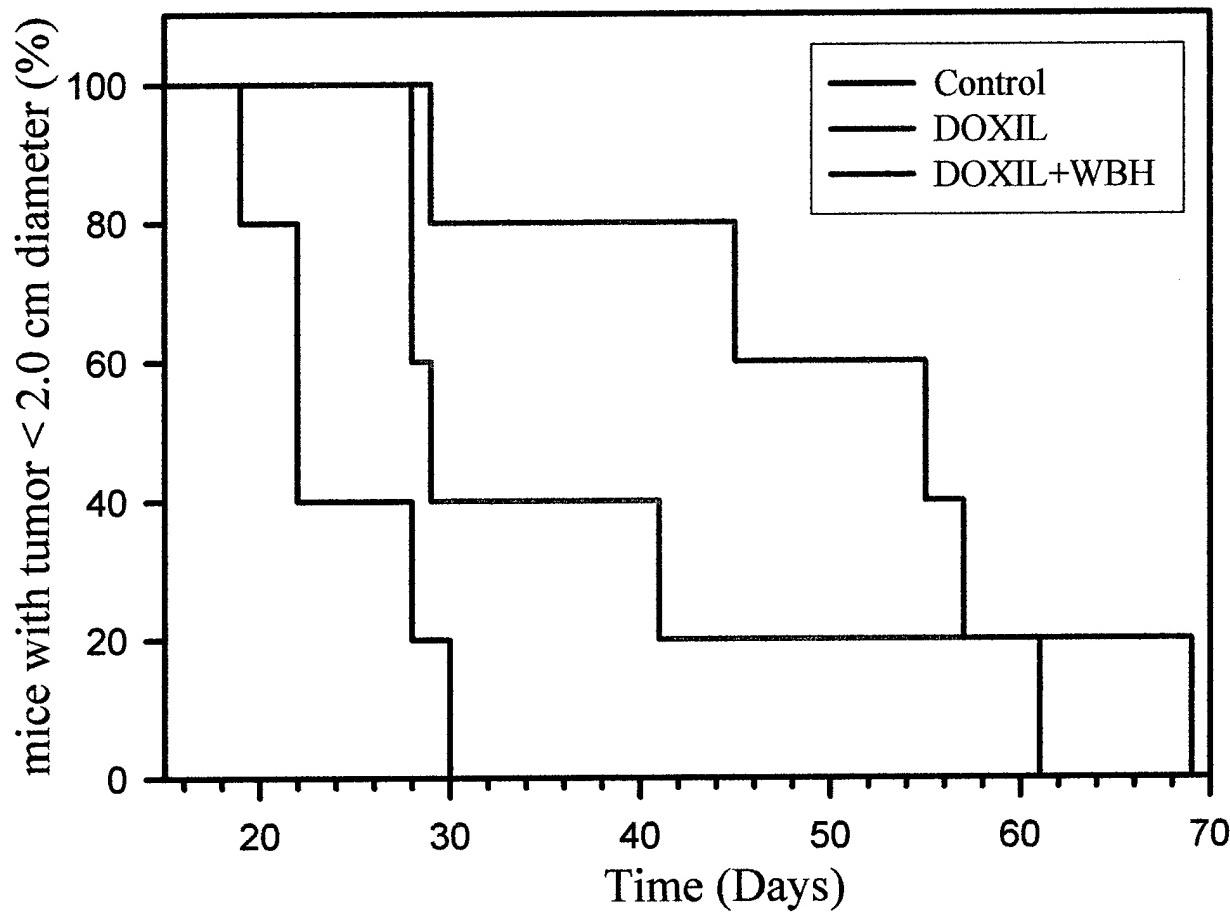
**Figure 5 A.** As we had observed in the past, the kinetic data gave very little support to combining free IL-12 with WBH. We concluded that the IL-12 treatment should cease, and that the mice should be observed and sacrificed only when their tumors reached 1500mm<sup>3</sup> in diameter.

- Figure 5 B.** Combination of IL-12 with WBH resulted in a survival advantage. One mouse in this group was able to completely reject its tumor. Overall, we concluded that there is a benefit to evaluating immunotherapies in immunocompetent mice instead of in immunodeficient mice.
- Figure 6 A.** Here we wished to optimize our treatment protocol. In other words, what is the best temporal placement of WBH with respect to that of the initiation of IL-12 treatment. Once again, the kinetic data appear to show that IL-12 is equally as effective alone as it is with WBH. We concluded that the IL-12 treatment should cease and that the mice should be observed and sacrificed only when their tumors reached  $1000\text{mm}^3$ .
- Figure 6 B.** We conclude from the Kaplan-Meier analysis that a survival advantage was evident in the mice receiving IL-12 just after WBH. This advantage is lost when IL-12 was given beginning 12 hours post WBH and was significantly inhibited when IL-12 was given just prior to WBH. Timing is a very important issue to consider when designing immunotherapy protocols with WBH.
- Figure 7.** Tumors in the combination group ceased to grow after administration of IL-12 microspheres on day 1. Tumors in the IL-12 microsphere alone group began to show an anti-tumor effect only after 9 days; this was a transient effect as all tumors went on to progress. Three of 5 mice were cured of their disease in the combination group. We concluded that the combination of IL-12 microspheres + WBH provided the best anti-tumor effect. These data suggest that there may be a distinct advantage in encapsulating cytokines into microspheres. These dramatic results were the most promising overall in the past year.

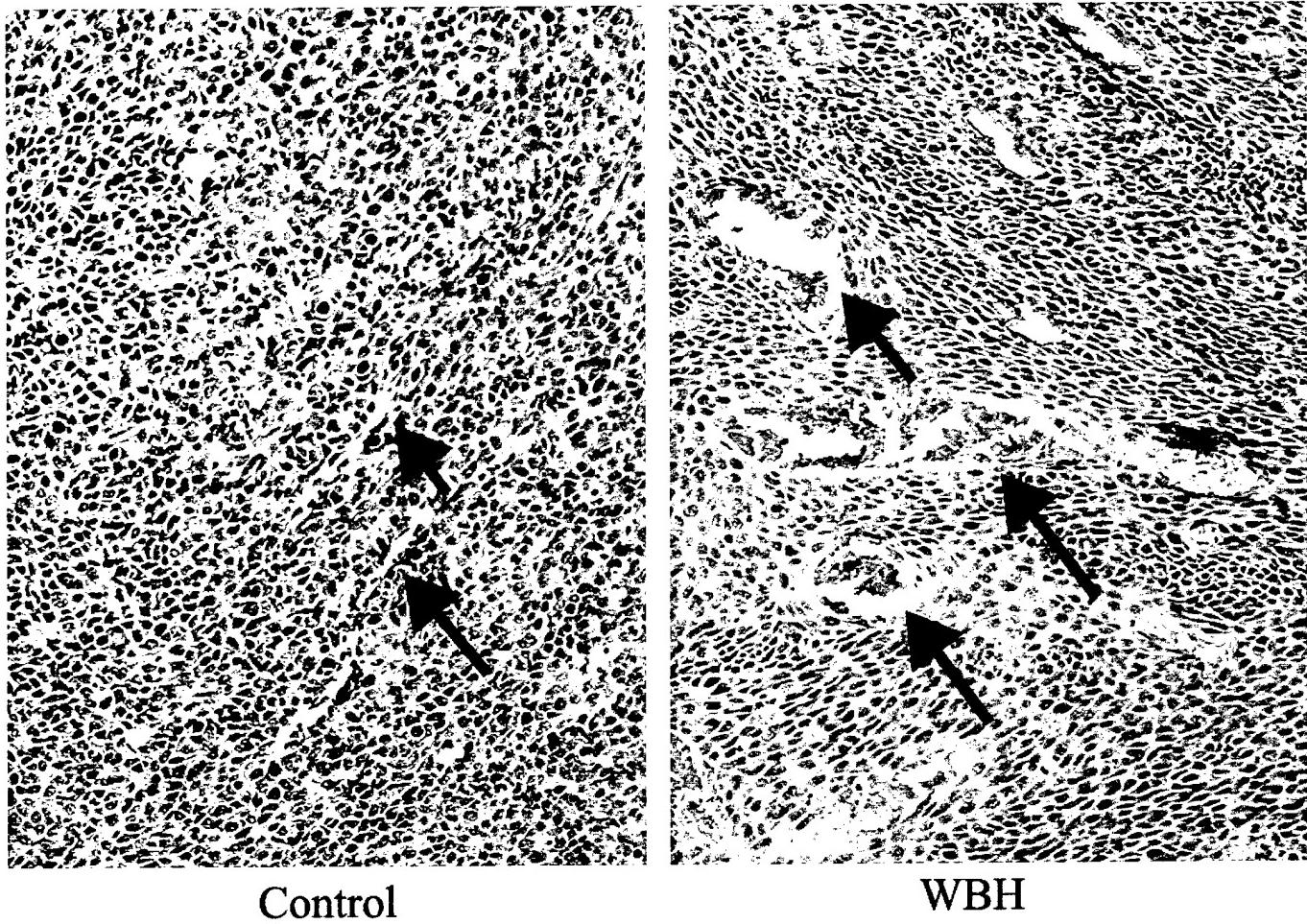
## APPENDIX



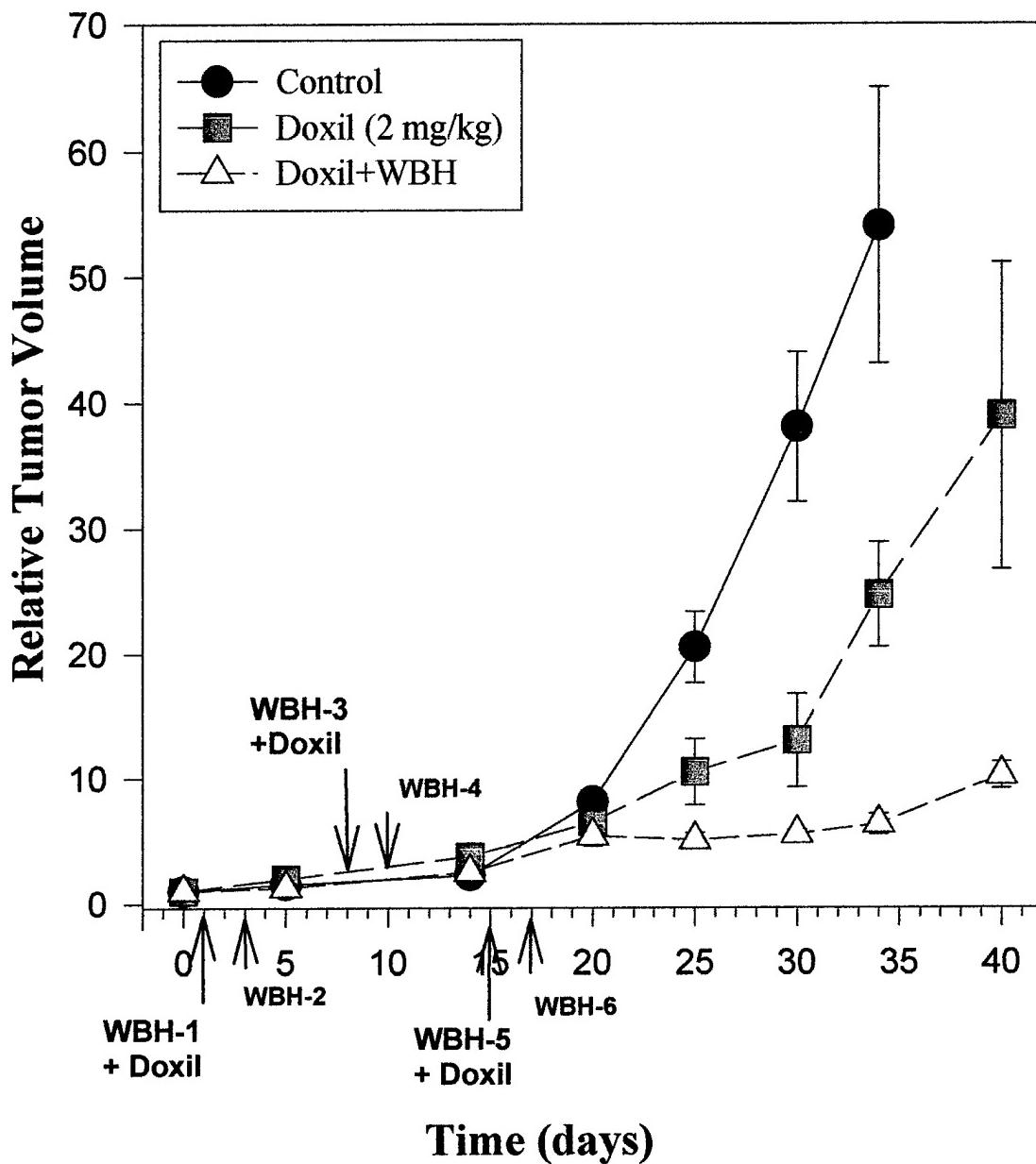
**Figure 1 A. WBH does not appear to enhance the anti-tumor effect of Doxil against human breast tumor #8990 in SCID mice (Day 0 – Day 20).** A human breast tumor was implanted s.c. in the lower left abdominal quadrant of female SCID mice. When the tumors reached 5 x 6mm, certain animals received WBH treatment (6 hours of a 2°C increase in body temperature;  $39.8 \pm 0.2^\circ\text{C}$ ). Immediately after WBH, 2mg/kg Doxil was administered, i.v. Control animals were given an i.v. injection of sterile 0.9% saline. A second WBH was administered on Day 10 preceding another dose of Doxil. Tumors were measured using digital Vernier calipers. Relative tumor volume was determined by dividing the average tumor volume per group on any given day by the average tumor volume of that same group on Day 0. ( $n = 5$ )



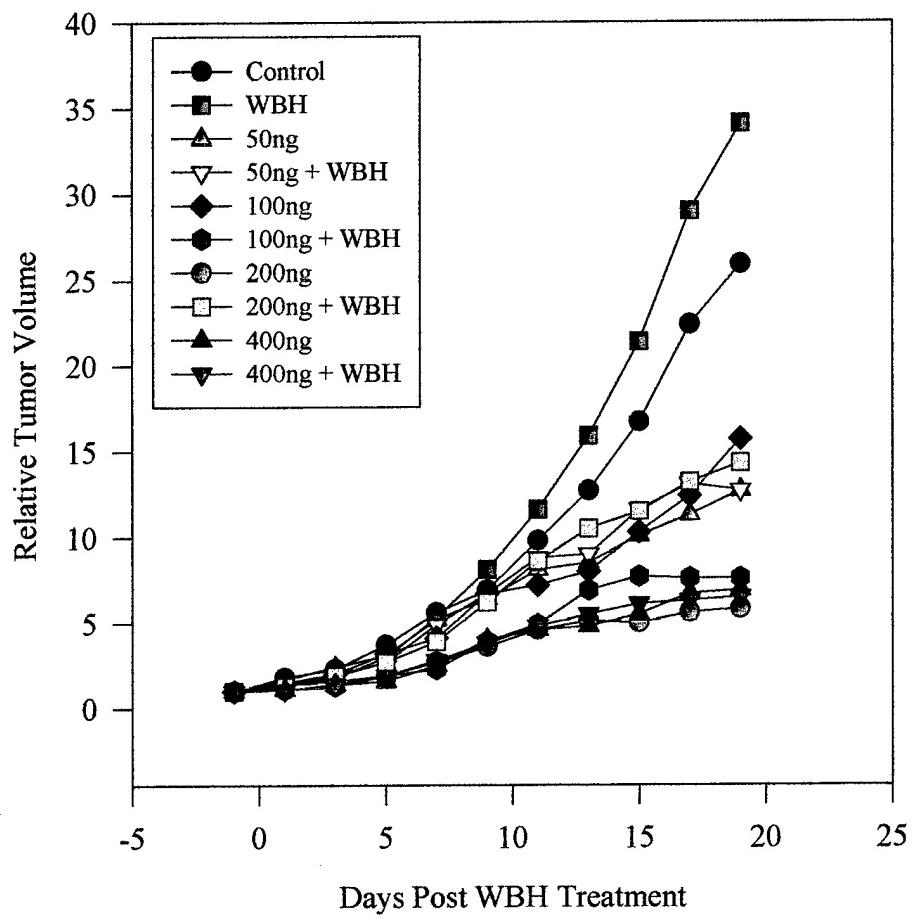
**Figure 1 B. WBH enhances the anti-tumor effect of Doxil against human breast tumor #8990 in SCID mice (Day 0 – Day 69).** A human breast tumor was implanted s.c. in the lower left abdominal quadrant of female SCID mice. When the tumors reached 5 x 6mm, certain animals received WBH treatment ( $39.8 \pm 0.2^\circ\text{C}$  for 6 hours). Immediately after WBH, 2mg/kg Doxil was administered, i.v. Control animals were given an i.v. injection of sterile 0.9% saline. A second WBH was administered on day 10 preceding another dose of Doxil. Tumors were measured using digital Vernier calipers. The data were graphed in a Kaplan-Meier plot to examine the time it took for tumors to attain a diameter of 2.0 cm. (n = 5)



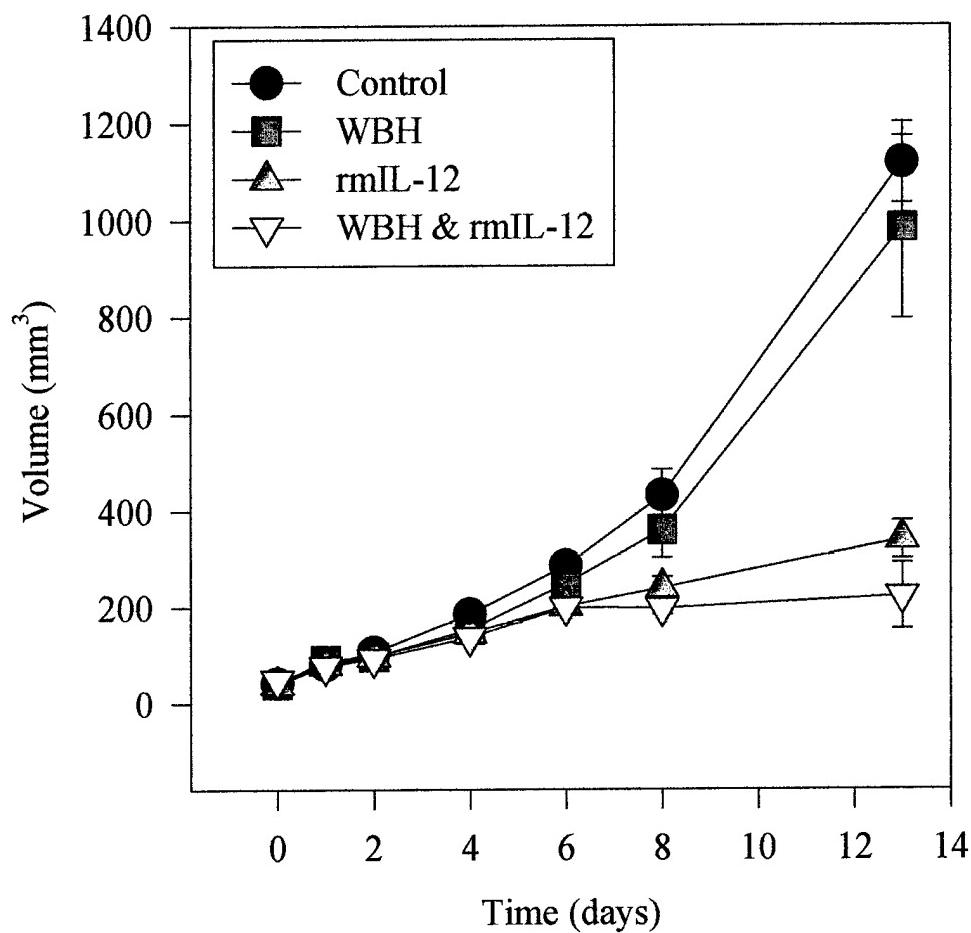
**Figure 2. WBH induces the enlargement of tumor blood vessels.** Female BALB/c mice bearing CT26 were treated with WBH (core body temperature of  $39.8 \pm 0.2^\circ\text{C}$  maintained for 6 hours). Two weeks later, the animals were sacrificed and their tumors were fixed in 10% buffered formalin, embedded in paraffin and sectioned for histological analysis. Sections are representative of 4 tumors analyzed from both control and WBH treated mice. (H&E, 100x magnification)



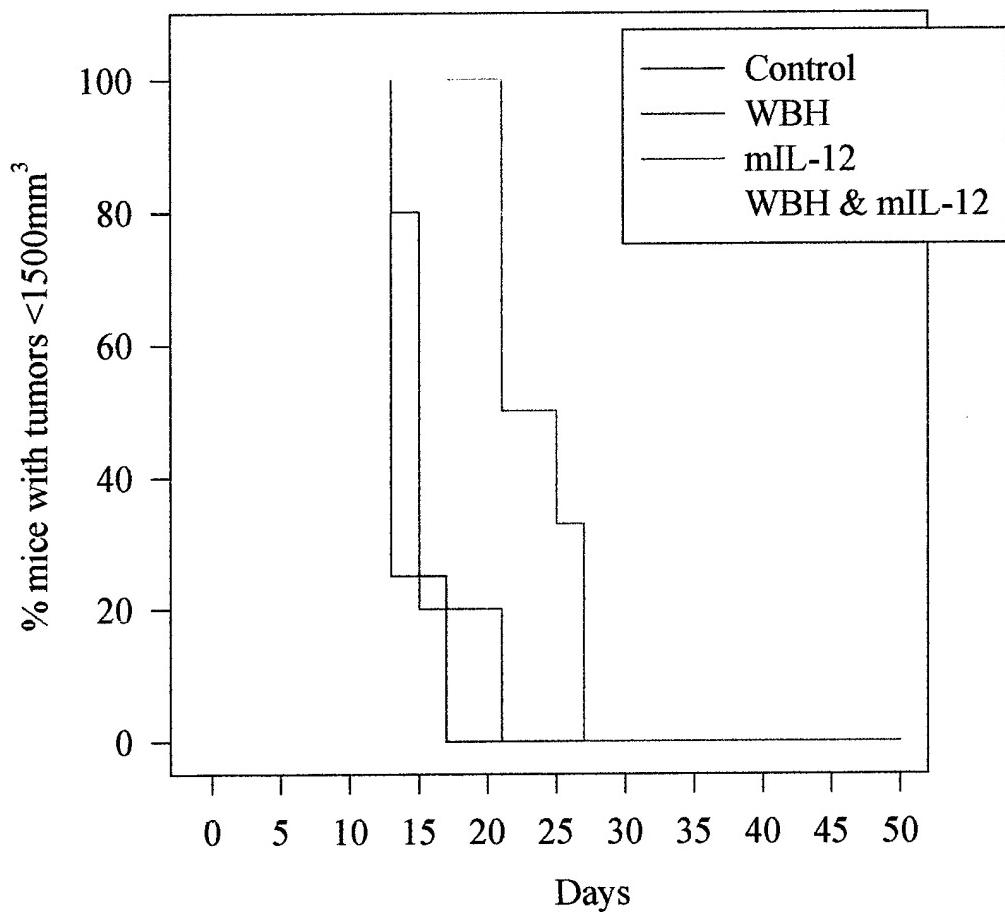
**Figure 3. WBH + Doxil greatly enhances the anti-tumor effect of Doxil alone.** SCID mice bearing human breast tumor #10366 were treated with Doxil with or without WBH in three identical cycles 1 week apart for 3 weeks. Tumor growth was measured using digital Vernier calipers. (n = 5)



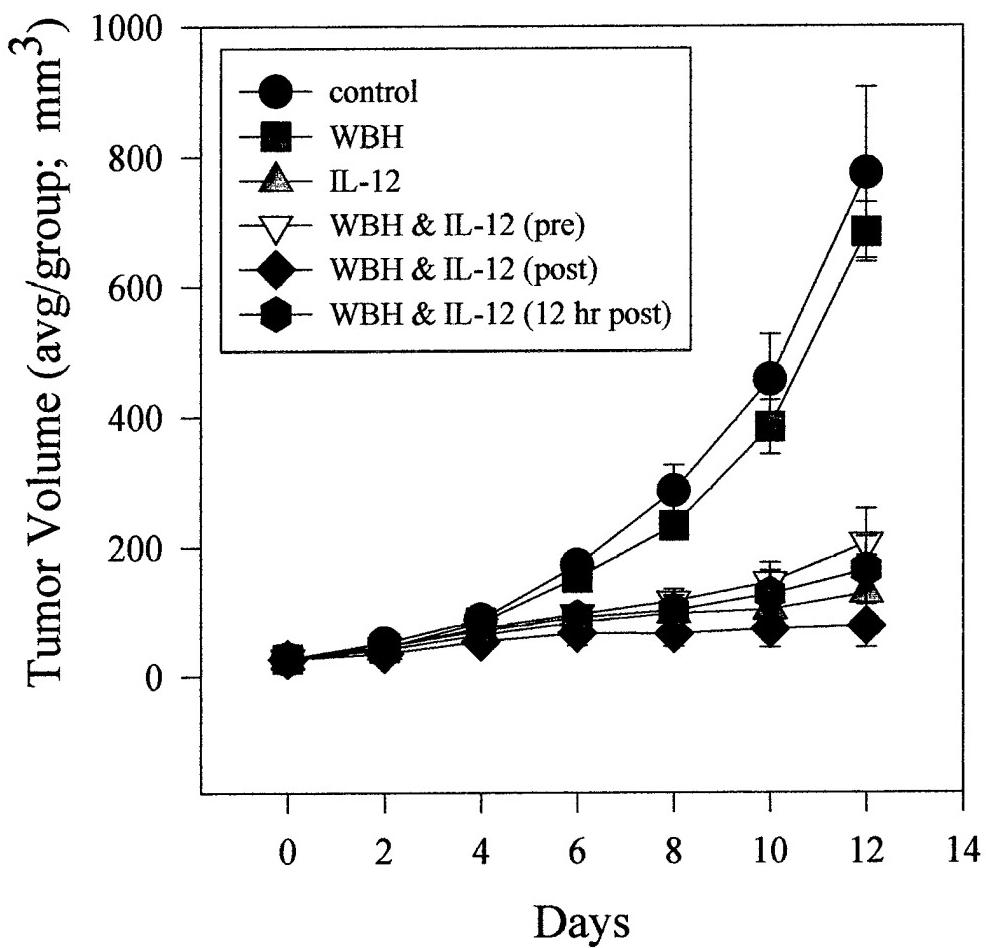
**Figure 4. WBH slightly enhances the anti-tumor effect of IL-12-based immunotherapy in SCID mice bearing a human breast tumor.** SCID mice bearing human breast tumor #8990 were given a single treatment of WBH on Day 1. Fifty, 100, 200 or 400 ng of free IL-12 was administered i.p. beginning on Day 1 (post WBH) up to and including Day 20. Only the 100 ng/day dose of IL-12 appears to be enhanced by WBH. (n = 2)



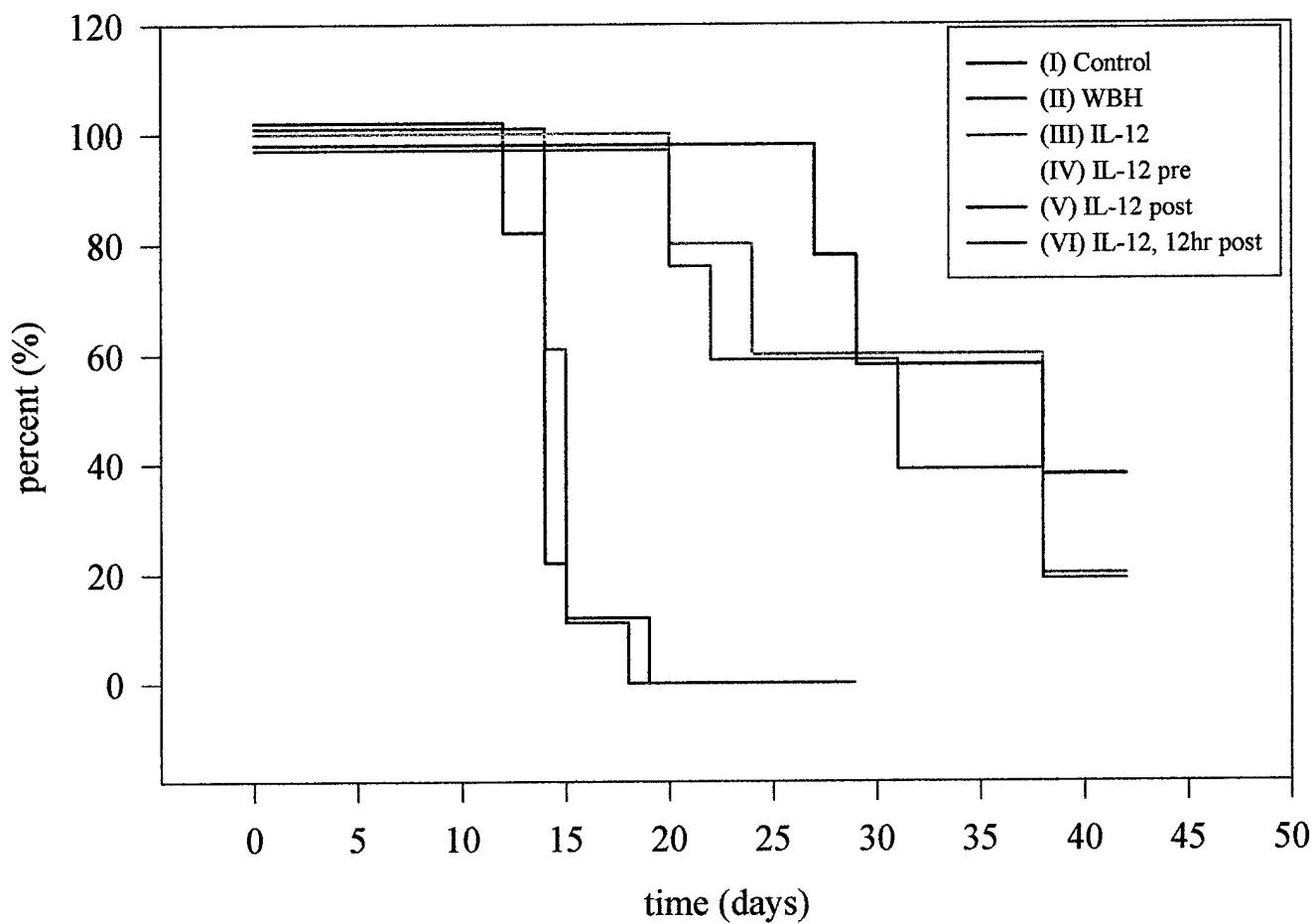
**Figure 5 A. WBH does not appear to enhance the anti-tumor effect of IL-12 immunotherapy in BALB/c mice bearing CT26 tumors (Day 0 – Day 13).** Female BALB/c mice bearing CT26 tumors were treated with WBH ( $39.8 \pm 0.2^\circ\text{C}$  for 6 hours). After WBH, IL-12 treatment (100 ng/mouse) was initiated and continued for 13 days. Tumors were measured using digital Vernier calipers. Tumor volume was calculated as the smallest tumor diameter (a) squared multiplied by the longest diameter (b) multiplied by 0.4.  $[(a^2)(b)(0.4) = \text{tumor volume, mm}^3]$  A. tumor growth analysis during IL-12 treatment. B. Time for each tumor to reach a volume of  $1500\text{mm}^3$ . One cure was achieved and this was in the combination group. ( $n = 4-5$  per group)



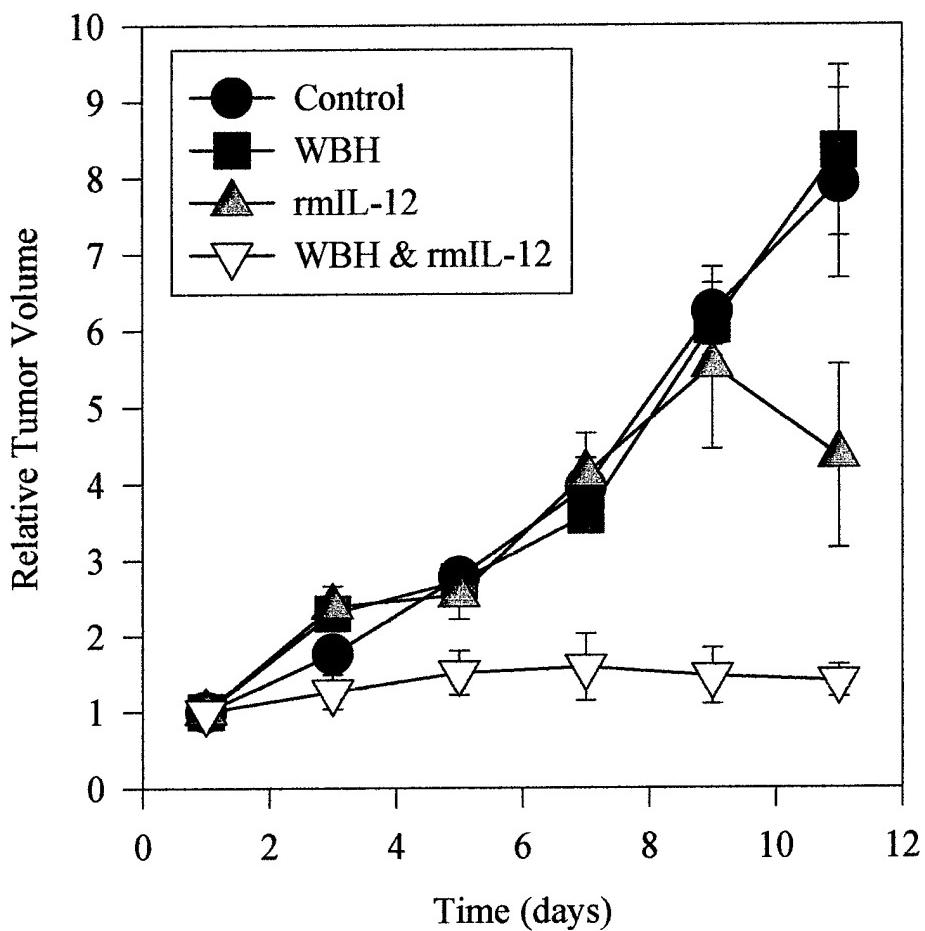
**Figure 5 B. WBH enhances the anti-tumor effect of IL-12 immunotherapy in BALB/c mice bearing CT26 tumors (Day 0 – Day 50).** Female BALB/c mice bearing CT26 tumors were treated with WBH ( $39.8 \pm 0.2^\circ\text{C}$  for 6 hours). After WBH, IL-12 treatment (100 ng/mouse) was initiated and continued for 13 days. Tumors were measured using digital Vernier calipers. Animals were sacrificed when their tumors reached volume of 1500mm<sup>3</sup>. Tumor volume was calculated as the smallest tumor diameter (a) squared multiplied by the longest diameter (b) multiplied by 0.4.  $[(a^2)(b)(0.4)] = \text{tumor volume, mm}^3$



**Figure 6 A. Timing of WBH with respect to IL-12 treatment does not appear to alter the anti-tumor effect of IL-12 immunotherapy in BALB/c mice bearing CT26 tumors (Day 0 – Day 12).** Female BALB/c mice bearing CT26 tumors were given 100 ng of IL-12 immediately before, 15 minutes after or 12 hours after WBH treatment ( $39.8 \pm 0.2^\circ\text{C}$  for 6 hours). IL-12 was administered daily for 12 days. (n = 5)



**Figure 6 B. Correct temporal placement of WBH with respect to that of IL-12 treatment is essential for enhancement of the anti-tumor effect of IL-12 alone (Day 0 – Day 42).** Female BALB/c mice bearing CT26 tumors were given 100 ng of IL-12 immediately before, 15 minutes after or 12 hours after WBH treatment ( $39.8 \pm 0.2^\circ\text{C}$  for 6 hours). IL-12 was administered daily for 12 days. Animals were sacrificed when their tumors reached a volume of  $1000\text{mm}^3$ . Two cures were achieved in the IL-12 (post) group, 1 cure in both the IL-12 alone and IL-12 (12 hours post) groups while no cures were achieved in the IL-12 (pre) group. ( $n = 5$ )



**Figure 7. WBH enhances the anti-tumor effect of polyactic acid encapsulated IL-12.** Female BALB/c mice bearing CT26 tumors were treated with WBH ( $39.8 \pm 0.2^\circ\text{C}$ ) for 6 hours. Fifteen minutes after WBH, mice were given a single, intratumoral injection of IL-12 loaded polyactic acid microspheres (0.2mg). Tumors were measured using digital Vernier calipers and relative volume assessed by the following formula,  $(a^2)(b)(0.4) = \text{tumor volume, mm}^3$ . Three of 5 mice in the combination group were cured of their disease. ( $n = 5$ )